

Glycogen synthase kinase 3 β together with 14-3-3 protein regulates diabetic cardiomyopathy: Effect of losartan and tempol

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Abstract Glycogen synthase kinase (GSK) 3 β is a multifunctional protein that positively regulates myocardial apoptosis and negatively regulates hypertrophy. However, the role of GSK3 β in the diabetic myocardium is largely unknown. We found that GSK3 β became more active (less phosphorylated at serine 9) via decreased Akt phosphorylation, in parallel to c-Jun NH₂ terminal kinase activation, which correlated with increased activated caspase 3 and myocardial apoptosis 3 days after streptozotocin (STZ) injection in mice. However, 28 days after STZ injection, GSK3 β became inactive, which correlated with the enhanced protein kinase C β 2 and p38 mitogen activated protein kinase expression, nuclear translocation of nuclear factor of activated T cells c3, cardiac hypertrophy and fibrosis. All of the above parameters were exacerbated in dominant-negative 14-3-3 transgenic mice. Our results suggest that GSK3 β together with 14-3-3 protein plays essential roles in the signaling of diabetic cardiomyopathy, and treatment with either losartan or tempol prevents these changes.

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1. Introduction

Glycogen synthase kinase (GSK) 3 β is a multifunctional protein, and is normally active in cells [1]. However, its increased activation has been shown to induce myocardial apoptosis [2]. Protein kinase B also called Akt, phosphorylates GSK3 β at serine 9, and GSK3 β thereby becomes inactive,

which leads to cardiac hypertrophy [3]. Recently, it has been demonstrated that inhibition of 14-3-3 protein causes increased phosphorylation of serine 9 and inactivation of GSK3 β [4]. Diabetic cardiomyopathy is associated with cardiac apoptosis, hypertrophy and fibrosis [5,6]. However, the role of GSK3 β in diabetic cardiomyopathy is largely unknown. The present study is sought to examine whether GSK3 β plays a role in mediating cardiac apoptosis and hypertrophy in the diabetic myocardium. The relationship between GSK3 β and 14-3-3 protein in the diabetic myocardium was further examined by using transgenic (TG) mice with cardiac-specific overexpression of a dominant-negative (DN) mutant of 14-3-3 protein.

Diabetic myocardium is associated with the increased levels of angiotensin II and oxidative stress [7–9]. Current treatments for diabetes-associated cardiomyopathy consist of β -blockers, thiazolidinediones and angiotensin II converting enzyme inhibitors [10]. Clinically, the effect of angiotensin II type 1 (AT1) receptor blockers in diabetic cardiomyopathy are not well understood. Recent studies have demonstrated that treatment with antioxidants inhibits hypertrophic responses of cardiac myocytes [11]. The present study examined the effects of treatment with losartan, an AT1 receptor blocker, and tempol, a superoxide dismutase mimetic, and attempted to clarify their molecular signaling mechanisms in mediating diabetes-induced myocardial apoptosis, hypertrophy and fibrosis.

The results of our study show that GSK3 β together with 14-3-3 protein regulates the signaling in diabetes-induced myocardial apoptosis, hypertrophy and fibrosis. Also, treatment with either losartan or tempol significantly attenuated diabetes-induced myocardial apoptosis, hypertrophy and fibrosis through improving GSK3 β -mediated signaling and associated events.

2. Materials and methods

2.1. Materials

Losartan was kindly donated by Banyu Pharmaceuticals, Tokyo, Japan. Tempol was purchased from Wako Chemicals, Osaka, Japan. Streptozotocin (STZ) was purchased from Sigma–Aldrich Inc., St. Louis, MO, USA. Polyclonal angiotensin II antibody was obtained from Peninsula Laboratories Inc., San Carlos, CA, USA. Monoclonal phospho-Akt (Ser 473), polyclonal Akt, polyclonal phospho GSK3 β (Ser 9), polyclonal GSK3 β , polyclonal cleaved caspase 3, polyclonal phospho-p38 mitogen activated protein kinase (MAPK) (Thr 180/Tyr 182), polyclonal p38 MAPK, polyclonal phospho-c-Jun NH₂ terminal kinase (JNK), and polyclonal JNK antibodies were obtained

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Abbreviations: ANP, natriuretic peptide; AT1, angiotensin II type 1; BW, body weight; DN, dominant negative; GSK, glycogen synthase kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HW, heart weight; HW/BW, the ratio of HW to BW; JNK, c-Jun NH₂ terminal kinase; MAPK, mitogen activated protein kinase; NFAT, nuclear factor of activated T cells; NTG, non-transgenic; PKC, protein kinase C; S.E.M., standard error mean; STZ, streptozotocin; TBS, Tris-buffered saline; TGF, transforming growth factor; TG, transgenic

from Cell Signaling Technology Inc., Beverly, MA, USA. Polyclonal AT1 receptor, polyclonal p22 phox, polyclonal nuclear factor of activated T cells (NFAT) c3, polyclonal atrial natriuretic peptide (ANP), polyclonal transforming growth factor (TGF) β 1, polyclonal collagen III, polyclonal protein kinase C (PKC) β 2 and polyclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.

2.2. Generation of DN-14-3-3 TG mice

DN-14-3-3 TG mice were generated as described previously [12]. In brief, the coding region of the human DN (R56A and R60A) 14-3-3 η cDNA with a 5'-Myc-1 epitope tag was subcloned into a vector containing the α -myosin heavy chain promoter and an SV40 polyadenylation site. Linearized DNA was injected into the pronuclei of one-cell C57BL/6 XSJL embryos at the Neuroscience Transgenic Facility at Washington University School of Medicine. Progeny were backcrossed into the C57BL/6 genetic background and were analyzed by polymerase chain reaction to detect transgene integration using mouse-tail DNA as template. Age-matched C57BL/6 JAX mice (obtained from Charles River Japan Inc., Kanagawa, Japan) were used as non-transgenic (NTG) controls.

2.3. Diabetes induction

STZ was administered by a single intraperitoneal injection at the dose of 150 mg/kg body weight (BW) to 8–10-week-old male DN-14-3-3 and NTG mice. STZ was dissolved in 20 mM sodium citrate saline buffer (pH 4.5) and injected within 5 min of preparation. Age-matched DN-14-3-3 and NTG mice were injected with 100 μ l of citrate buffer and used as non-diabetic controls. Mice were maintained with free access to water and chow throughout the period of study, and the animals were treated in accordance with the guidelines for animal experimentation of our institute.

2.4. Experimental protocol

Animals were studied at two times after streptozotocin administration, i.e., on day-3 for acute study and on day-28 for chronic study. For each of these examinations times, both TG and NTG mice were divided into the following 6 groups: (1) vehicle-treated normal (non-STZ-induced) group (N), (2) STZ-induced and vehicle-treated group (D), (3) STZ-induced and losartan-treated group (D + L), (4) STZ-induced tempol-treated group (D + T), (5) Losartan-treated normal group, and (6) Tempol-treated normal group. For the 3-day time-period study, treatment with either losartan or tempol was started from 3 days before the administration of STZ, and continued up to 3 days after STZ injection. Losartan (60 mg/kg/day) was dissolved in water and given orally once a day, while tempol (1.5 mmol/kg/day) was dissolved in normal saline and administered intraperitoneally once a day. For the 28-day time-period study, either losartan or tempol was started from 24 h before the administration of STZ, and continued for 28 days after STZ injection. Losartan (12 mg/kg/day) and tempol (1 mmol/L) were dissolved in drinking water and given ad libitum. Drug dosage and solvents for drugs were adopted from Refs. [13,14].

BWs and blood glucose levels of animals were measured before and at the end of each time-period protocol. Animals' blood glucose levels were determined using Medi-safe chips (Terumo Inc., Tokyo, Japan). At the end of each protocol, mice were anesthetized with a single intraperitoneal injection of pentobarbital (50 mg/kg), and the hearts were excised. Heart weight (HW) and the ratio of HW to BW (HW/BW) were determined for each animal. The left ventricle was quickly dissected and cut into two parts. One part was immediately transferred into liquid nitrogen and then stored at -80°C for protein analysis. The other part was either stored in 10% formalin to make paraffin sections or stored at -80°C after the addition of Tissue-Tek OCT compound (Sakura Co. Ltd., Tokyo, Japan) to make frozen tissue sections.

2.5. Immuno-histochemistry for angiotensin II and NFATc3

Frozen cardiac tissue sections were fixed in acetone, and the slides were washed in Tris-buffered saline (TBS; 10 mmol/L Tris-HCl, 0.85% NaCl, pH 7.5) containing 0.1% BSA. Endogenous peroxidase activity was quenched by incubating the slides in methanol and 0.6% H_2O_2 in methanol. Blocking was done with normal serum. After overnight incubation with either anti-angiotensin II antibody (1:50 dilution) or anti-NFATc3 antibody (1:25 dilution) at 4°C , the slides were

washed in TBS buffer and HRP conjugated secondary antibody (Santa Cruz) was added and incubated at room temperature for 45 min. The immuno-staining was visualized using diaminobenzidine tetrahydrochloride the slides were counterstained with hematoxylin. A negative control without primary antibody was included in the experiment to ensure the antibody specificity. Brown colored immuno-positive angiotensin II spots were counted at 1000 \times magnification, and depicted as the average number of immuno-positive myocytes per field. Nuclear translocation of NFATc3 was measured by counting the average number of nuclei stained with anti-NFATc3 antibody per high power field (1000 \times). For both cases, 50 random fields representing the whole section were examined per section, and 3 animals were used per group.

2.6. Protein analysis by Western blotting

Protein lysate was prepared from heart tissue as described previously [15]. The total protein concentration in samples was measured by a modified Lowry method. For Western blotting experiments, 100 μ g of total protein was loaded and proteins were separated by SDS-PAGE (200 V for about 40 min) and electrophoretically transferred to nitrocellulose filters (semi-dry transfer at 10 V for 30 min). Filters were blocked with 5% non-fat dry milk in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl and 0.1% Tween 20). All antibodies were used at a dilution of 1:1000. After incubation with primary antibody, bound antibody was visualized with horseradish peroxidase-coupled secondary antibody (Santa Cruz), and chemiluminescence developing agents (ECL Plus, Amersham). The expression of phospho-Akt, phospho-GSK3 β , phospho-p38 MAPK and phospho-JNK was normalized by the basal level of Akt, GSK3 β , p38 MAPK and JNK, respectively. The level of expression of AT1 receptor, p22 phox, activated caspase, ANP, TGF β 1, collagen III and PKC β 2 was normalized by GAPDH protein expression in the same sample. The level of expression of each protein in normal NTG mice was taken as one arbitrary unit. For Western blotting analysis, all primary antibodies were used at a dilution of 1:1000 and secondary antibodies were used at a dilution of 1:5000.

2.7. TUNEL apoptosis analysis

Frozen left ventricular (LV) tissues embedded in OCT compound were cut into 4- μ m thick sections and fixed in 4% paraformaldehyde (pH 7.4) at room temperature. TUNEL analysis was performed as specified in the in situ apoptosis detection kit (Takara Bio Inc., Shiga, Japan). Sections were mounted and examined using light microscopy. For each animal, five sections were scored for apoptotic nuclei. Only nuclei that were clearly located in cardiac myocytes were scored.

2.8. Myocardial cell size measurement

Paraffin-embedded LV tissue sections stained with hematoxylin-eosin, were used for measuring cell size. The short axis diameter of cardiac myocytes was measured for 10 myocytes selected per field (about 50 fields were selected per section) at 400-fold magnification by light microscopy. Each average value was obtained based on the data from 10 myocytes and was used as an independent sampling value.

2.9. Measurement of myocardial fibrosis

The area of myocardial fibrosis in LV tissue sections stained with Azan-Mallory was quantified using a color image analyzer (CIA-102, Olympus, Tokyo, Japan), based on the difference in color (blue fibrotic area as opposed to red myocardium) [16].

2.10. Statistical analysis

Data are presented as means \pm standard error mean (S.E.M.). Statistical analysis of differences between groups was performed by one-way analysis of variance followed by Tukey's method using JSTAT software. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of treatment with losartan or tempol on blood glucose, BW, HW and HW/BW

Treatment of STZ-induced diabetic mice with losartan or tempol did not alter the hyperglycemic state of the animals in

Table 1

Changes in blood sugar level, body and heart weights and HW/BW in mice with or without the treatment of either losartan or tempol for 28 days

	NTG				TG			
	N	D	D + L	D + T	N	D	D + L	D + T
Blood sugar level (mg/dl)	155 ± 28	489 ± 31**	531 ± 18**	528 ± 21**	157 ± 25	501 ± 12###	497 ± 19###	512 ± 19###
Body weight (g)	23.8 ± 0.98	20.1 ± 0.48*	22.1 ± 0.41	21.1 ± 0.21	25.4 ± 0.64	22.2 ± 0.48#	23.8 ± 0.61	22.9 ± 0.39
Heart weight (mg)	102 ± 4.1	93 ± 5.2*	96 ± 4.7	96 ± 4.1	105 ± 6.5	97 ± 5.1#	101 ± 5.5	98 ± 4.5
HW/BW (mg/g)	4.31 ± 0.32	4.36 ± 0.59	4.34 ± 0.52	4.33 ± 0.47	4.14 ± 0.46	4.31 ± 0.51#	4.25 ± 0.43	4.24 ± 0.44

Values are means ± S.E.M. HW/BW, the ratio of heart weight to body weight; N, normal (non-STZ-induced, age matched); D, STZ-induced diabetic; D + L, STZ-induced diabetic, treated with losartan; D + T, STZ-induced diabetic treated with tempol (for drug treatment protocol, see Section 2). * $P < 0.05$, ** $P < 0.01$ vs. N of NTG mice; # $P < 0.05$, ### $P < 0.01$ vs. N of TG mice. The results are means ± S.E.M. from 6 animals per group.

either the 3-day or 28-day time-period group, i.e., blood glucose levels remained elevated in the losartan-treated and tempol-treated groups. The BW, HW and HW/BW parameters were not significantly changed in normal animals (non-STZ induced) treated with losartan or tempol compared to those in normal animals (no drug treatment). Therefore, the BW, HW and HW/BW data of normal animals treated with losartan and tempol are not shown. STZ administration elevated the blood glucose level 3 days after STZ injection, and treatment period of STZ-induced diabetic mice with either losartan or tempol for 3-days did not show any significant changes in BW, HW or HW/BW compared to those in normal (non-STZ administered) and STZ-administered animals, so these data are not shown. Treatment with either losartan or tempol for a 28-day time period slightly improved the BW and HW (Table 1). HW/BW was significantly increased 28 days after STZ injection in the TG group relative to the age-matched normal animals, and the HW/BW was improved after treatment with either tempol or losartan, while the HW/BW did not differ significantly among NTG groups (Table 1). Animals which had blood glucose level higher than 300 mg/dl were used for further experiments. The results are means ± S.E.M. from 6 animals per group.

3.2. STZ injection induces myocardial angiotensin II and oxidative stress

The number of angiotensin II-positive cardiac myocytes was elevated in both TG and NTG mice 3 days (Fig. 1A and B) and 28 days (Fig. 1C) after STZ injection relative to that in age-matched normal animals. Furthermore, the expression of AT1 receptor was significantly upregulated in both TG and NTG mice 3 days (Fig. 1D and F) and 28 days (Fig. 1E and H) after STZ injection. However, the number of angiotensin-II positive cardiac myocytes and the expression of AT1 receptor did not differ significantly between TG and NTG mice 3 and 28 days after STZ injection. Treatment with either losartan or tempol significantly attenuated the expression of myocardial angiotensin II and AT1 receptor 3 days and 28 days after STZ injection (Fig. 1A–H).

Myocardial expression of p22 phox, a component of NADPH oxidase, was elevated in both TG and NTG mice 3 days (Fig. 1D and G) and 28 days (Fig. 1E and I) after STZ injection. However, the expression of p22 phox was significantly higher in TG mice than in NTG mice on both day 3 (Fig. 1D and G) and 28 (Fig. 1E and I) after STZ injection. The results are means ± S.E.M. from 3–5 experiments.

3.3. GSK3 β signaling in STZ-induced myocardial apoptosis

The phosphorylation of Akt and GSK3 β was significantly decreased in the myocardium of both TG and NTG mice 3

days after STZ injection relative to that in age-matched normal animals (Fig. 2A–C). These results are in accord with the increased expression of activated caspase 3 (Fig. 2A and D) and increased myocardial apoptosis (Fig. 2E and F) 3 days after STZ injection in both TG and NTG mice. However, a significant decrease in the phosphorylation of Akt and GSK3 β , but an increase in the expression of activated caspase 3 and in myocardial apoptosis were found in TG mice relative to NTG mice 3 days after STZ injection (Fig. 2A–F). Treatment with either losartan or tempol significantly improved the phosphorylation status of Akt and GSK3 β , and attenuated the expression of activated caspase and myocardial apoptosis in both TG and NTG mice 3 days after STZ injection (Fig. 2A–F). The results are means ± S.E.M. from 3–5 experiments. Myocardial apoptosis and the expression of activated caspase were did not differ significantly before and 28 days after STZ injection (data not shown).

3.4. GSK3 β phosphorylation and NFATc3 nuclear translocation

The phosphorylation of Akt did not differ significantly among groups 28 days after STZ injection (Fig. 3A and C). However, the phosphorylation of GSK3 β was slightly elevated in NTG mice but significantly elevated in TG mice 28 days after STZ injection relative to that in the age-matched normal animals (Fig. 3A and D). The increased phosphorylation of GSK3 β was accompanied by increased expression of PKC β 2 28 days after STZ injection (Fig. 3A and B). These results are in accord with the nuclear translocation of NFATc3, a transcription factor involved in cardiac hypertrophy [17]. While NFATc3-positive nuclei were significantly elevated 28 days after STZ injection in both TG and NTG mice (Fig. 3E and F), the average number of nuclei stained with anti-NFATc3 antibody was significantly higher in TG mice relative to NTG mice 28 days after STZ injection (Fig. 3E and F). Treatment with either losartan or tempol significantly attenuated the expression of PKC β 2, the phosphorylation of GSK3 β and the nuclear translocation of NFATc3 in both TG and NTG mice 28 days after STZ injection (Fig. 3A–F). The results are means ± S.E.M. from 3–5 experiments.

3.5. Treatment with losartan or tempol attenuates diabetes-induced myocardial hypertrophy and fibrosis

The average cross-sectional diameter of cardiac myocytes was slightly increased in NTG mice, but significantly increased in TG mice 28 days after STZ injection (Fig. 4A and C). At the same time, the % of myocardial fibrosis was significantly elevated in both TG and NTG mice 28 days after STZ injection (Fig. 4B and D). The expression of molecular markers of cardiac hypertrophy (ANP) and fibrosis (TGF β 1 and collagen III)

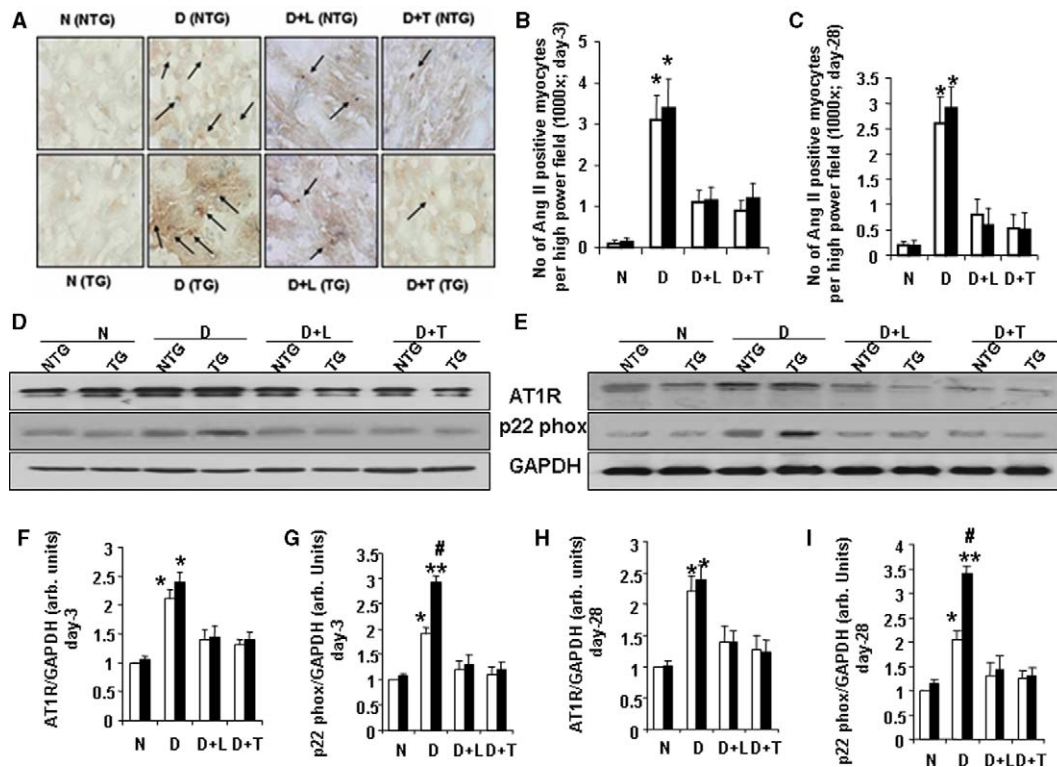


Fig. 1. Streptozotocin (STZ) injection elevates myocardial angiotensin II and oxidative stress. (A) Myocardial tissue sections performed with immuno-histochemical staining for angiotensin II, three days after STZ injection. NTG, non-transgenic; TG, transgenic. N, Normal (non-STZ-induced, age matched); D, STZ-induced diabetic; D + L, STZ-induced diabetic, treated with losartan; D + T, STZ-induced diabetic treated with tempol (for drug treatment protocol, see Section 2). Arrows indicate myocyte stained with angiotensin II. Magnification 1000 \times . (B, C) Quantification of angiotensin II positive myocytes per high power field at 1000 \times magnification on 3 days (B) and 28 days (C) after STZ injection. (D, E) Western-immunoblots showing the myocardial expression of angiotensin II type 1 (AT1) receptor and p22 phox 3 days (D) and 28 days (E) after STZ injection. GAPDH protein expression was used as control. Immunoblots are representative of three separate experiments. (F–I) Quantification of AT1 receptor on day-3 (F), p22 phox on day-3 (G), AT1 receptor on day-28 (H) and p22 phox on day-28 (I). Densitometric analysis of immuno-reactive bands was performed using Scion Image software. The results are means \pm S.E.M. from 3–5 experiments. Open bar, NTG; closed bar, TG. * P < 0.05, ** P < 0.01 vs. N, D + L and D + T of the same genetic group. # P < 0.05 vs. D of NTG group.

was also significantly elevated in both TG and NTG mice 28 days after STZ injection (Fig. 4E–H). Myocardial cell diameter, % of fibrosis and the expression of ANP, TGF β 1 and collagen III were significantly elevated in TG mice relative to NTG mice 28 days after STZ injection (Fig. 4A–H). Treatment with either losartan or tempol significantly attenuated the increase in myocardial cell diameter, cardiac fibrosis and the myocardial expression of ANP, TGF β 1 and collagen III in both TG and NTG mice 28 days after STZ injection (Fig. 4A–H). The results are means \pm S.E.M. from 3–5 experiments.

3.6. Treatment with losartan or tempol attenuates MAPK signaling in the diabetic myocardium

Previously, we showed that activation of JNK was increased on day 3 and decreased on day 28, whereas the activation of p38 MAPK was decreased on day 3, but increased on day 28 [15]. ERK 1/2 was not activated at either time [15]. In the present study, we examined the effect of treatment with either losartan or tempol on the JNK (using 3-day samples) and p38 MAPK (using 28-day samples) signaling. Our results showed that treatment with either losartan or tempol attenuated the myocardial activation of JNK and p38 MAPK at 3 days and 28 days after STZ injection, respectively, in both

TG and NTG mice (Fig. 5A–D). Data are presented as the mean signal intensity \pm S.E.M. from 3 experiments.

4. Discussion

Hyperglycemia activates the local renin angiotensin system, resulting in the formation of angiotensin II [7,8], and it has been shown in both clinical and experimental studies that angiotensin II induces oxidative damage by producing ROS through the NADH/NADPH oxidase system [18]. In addition, myocardial apoptosis has also been reported in both diabetic patients [5] and diabetic animals [7,8]. Although, activation of GSK3 β has been shown to induce myocardial apoptosis [2], the role of GSK3 β has been in the diabetic myocardium is largely unknown. GSK3 β is normally active in the cell, but becomes inactivated when phosphorylated at ser-9 by Akt [3]. In addition inactivation of GSK3 β is also correlated to myocardial hypertrophy [3]. In this study, we found that the phosphorylation of both Akt and GSK3 β at ser-9 was significantly decreased 3 days after STZ injection in the myocardium of diabetic animals compared to that in age matched normal animals, which indicates that GSK3 β activity is upregulated in the early stage of STZ-induced diabetic myocardium relative

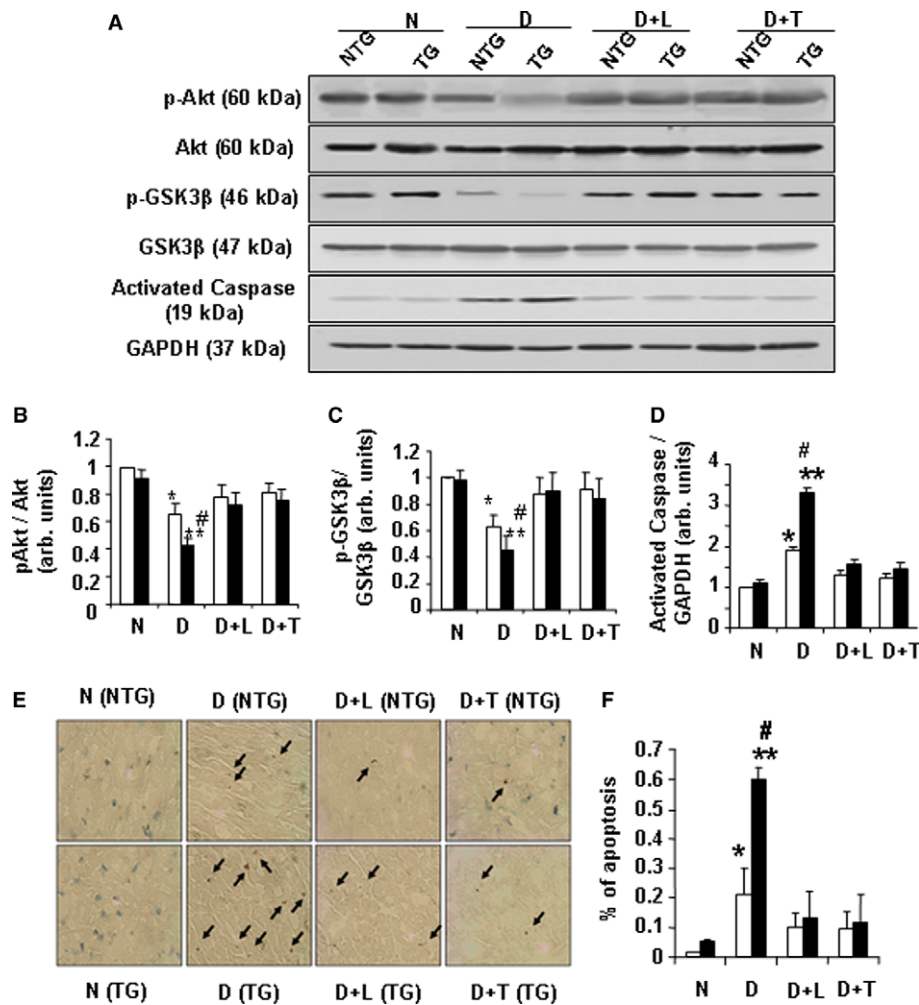


Fig. 2. GSK3 β signaling in streptozotocin-induced myocardial apoptosis. (A) Western-immunoblots showing the myocardial expression of phospho-Akt (p-Akt), Akt, phospho-GSK3 β (p-GSK3 β), GSK3 β and activated caspase 3 three days after STZ injection. GAPDH protein expression was used as control. These immunoblots are representative of three separate experiments. (For abbreviations, see legend of Fig. 1.) (B–D) Quantification of Akt (B) GSK3 β (C) and activated caspase 3 (D) three days after STZ injection. Densitometric analysis of immuno-reactive bands was performed using Scion Image software. (E) Myocardial tissue sections stained for TUNEL positive apoptotic nuclei (indicated by arrows) 3 days after STZ injection. Magnification 400 \times . (F) Quantification of % of TUNEL-positive apoptotic cardiac myocytes 3 days after STZ injection. The results are means \pm S.E.M. from 3–5 experiments. Open bar, NTG; closed bar, TG. * P < 0.05, ** P < 0.01 vs. N, D + L and D + T of the same genetic group. # P < 0.05 vs. D of NTG group.

to normal myocardium. Activation of caspase 3 is reported to be a downstream event in GSK3 β signaling in the mediation of myocardial apoptosis [19]. In this study, we examined myocardial apoptosis by assessing both caspase-3 activation and TUNEL staining. We found that expression of activated caspase 3 and the amount of myocardial apoptosis were significantly increased 3 days after STZ injection, and were correlated with the enhanced activation of GSK3 β . Our results suggest for the first time that GSK3 β plays an important role in mediating myocardial apoptosis in the diabetic myocardium. Since, insulin is reported to negatively regulate GSK3 β activity [1], we believe that STZ-induced lack of insulin may be one potential event resulting in the activation of GSK3 β in the early stage of experimental diabetes in the present study. Although the relationship between 14-3-3 protein and GSK3 β in the regulation of apoptosis is not well established, we hypothesize that on day 3 the pro-apoptotic signaling (GSK3 β) may exceed the anti-apoptotic signaling (14-3-3). However, further studies

will be required to confirm this. Previously, we showed that the activation of JNK was correlated with the myocardial apoptosis [15]. Very recently, Enguita et al. [20] reported that JNK as well as GSK3 β signaling pathways mediate cell death pathways. This suggested that, it was worthwhile to examine the roles of GSK3 β and JNK with respect to 14-3-3 protein in mediating myocardial apoptosis in the diabetic myocardium. It is interesting to note that the phosphorylation of GSK3 β was significantly decreased in the myocardium of DN-14-3-3 mice, compared to that of NTG mice 3 days after STZ injection, which shows that GSK3 β becomes more highly activated in DN-14-3-3 mice, in which about 50% of the function of pan-14-3-3 protein is inhibited as we demonstrated previously [12]. Interestingly, 14-3-3 has been shown to have a significant interaction with upstream effectors of both GSK3 β and JNK, such as Akt [21] and apoptosis signal regulating kinase-1 [22], respectively. Whether 14-3-3 protein prevents apoptosis in the diabetic myocardium by direct or indirect interaction with

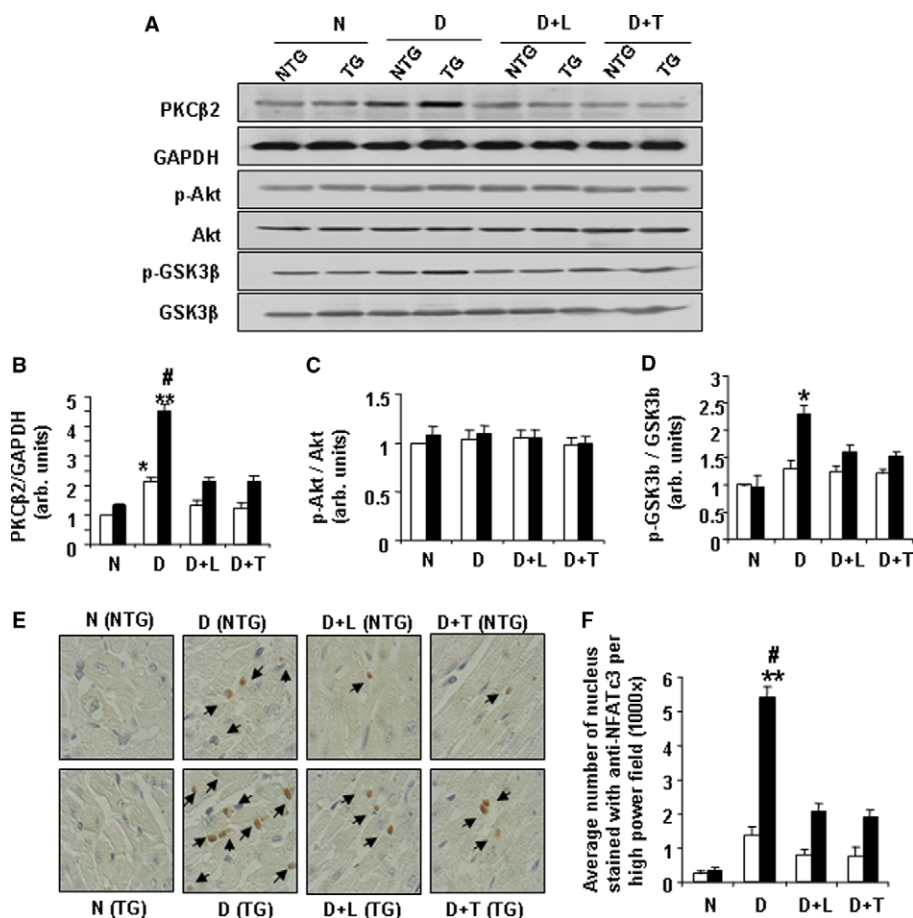


Fig. 3. GSK3β phosphorylation and NFATc3 nuclear translocation. (A) Western-immunoblots showing the myocardial expression of PKCβ2, phospho-Akt (p-Akt), Akt, phospho-GSK3β (p-GSK3β) and GSK3β 28 three days after STZ injection. These immunoblots are representative of three separate experiments. (For abbreviations, see legend of Fig. 1.) (B–D) Quantification of PKCβ2 (B), Akt (C) and GSK3β (D) 28 days after STZ injection. Densitometric analysis of immuno-reactive bands was performed using Scion Image software. (E) Myocardial tissue sections showing the nuclear staining for NFATc3 (indicated by arrows) 28 days after STZ injection. Magnification 1000x. (F) Quantification of the nuclear translocation of NFATc3 in cardiac myocytes 28 days after STZ injection. The results are means \pm S.E.M. from 3–5 experiments. Open bar, NTG; closed bar, TG. * $P < 0.05$, ** $P < 0.01$ vs. N, D + L and D + T of the same genetic group. # $P < 0.05$ vs. D of NTG group.

both GSK3β and JNK is still speculative at present and answering this question will require further research.

GSK3β has been shown to inhibit cardiac hypertrophy by preventing the nuclear translocation of NFAT3 via phosphorylation of NFAT3 [23]. NFATc3 is a transcription factor which activates several genes related to cardiac hypertrophy [17]. Phosphorylation of NFAT facilitates the binding of 14-3-3 protein to NFAT, and consequently NFATc3 is localized to the cytoplasm and the NFATc3-mediated transcription is inhibited [4]. In the present study, the phosphorylation of GSK3β and the nuclear translocation of NFATc3 were elevated in NTG mice, with a pronounced increase observed in DN-14-3-3 mice relative to NTG mice 28 days after STZ injection, without a significant alteration in Akt activity, suggesting that there may be other upstream activators of GSK3β which control its signaling in the diabetic myocardium converting the pro-apoptotic stimuli of GSK3β observed in early stages of the disease, and that these other activators were more active in DN-14-3-3 mice. In this study, as reported earlier [24], cardiac hypertrophy and its marker protein ANP, and cardiac fibrosis and its marker proteins TGFβ1 and collagen III, were significantly elevated in DN-14-3-3 mice relative to NTG mice 28

days after STZ injection (Fig. 4). Since NFATc3 is regulated by both GSK3β and 14-3-3 protein, the partial inactivation of 14-3-3 protein in DN-14-3-3 mice may have resulted in higher nuclear translocation of NFATc3 and exacerbation of cardiac hypertrophy in the myocardium of DN-14-3-3 mice relative to NTG mice 28 days after STZ injection. Our results are in accordance with the reported finding that shows the inhibition of pan-14-3-3 protein caused the phosphorylation and inactivation of GSK3β, which led to cardiac hypertrophy [4]. Although cardiac hypertrophy is often associated with fibrosis, as observed in the present study, the role of GSK3β in the induction of cardiac fibrosis still remains elusive. Laviola et al. [25] failed to observe a significant change in GSK3β phosphorylation after 6 weeks after of diabetes induction in rats. Although, this discrepancy might be due to species difference, it suggests that GSK3β seems to have multiple roles in the diabetic myocardium.

Recently, GSK3β was also shown to be inactivated by PKC [26], and several studies including the present study indicated that the PKCβ2 isoform is elevated in the diabetic myocardium [27]. It is intriguing to note that in the present study a parallel relationship was observed between the level

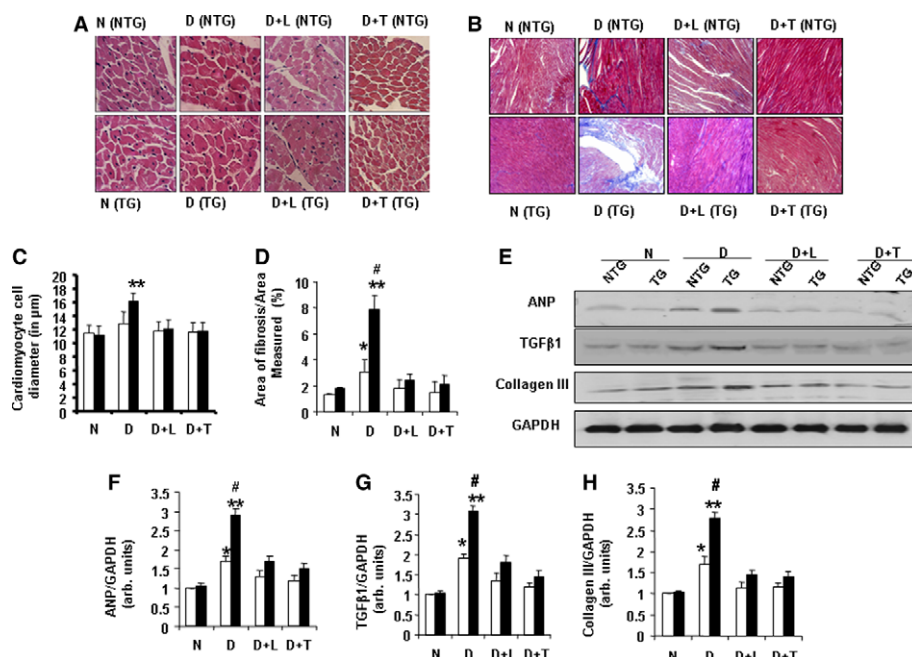


Fig. 4. Treatment with losartan and tempol attenuates STZ-induced cardiac hypertrophy and fibrosis. (A) Myocardial tissue sections obtained 28 days after STZ injection and stained with hematoxylin and eosin (H-E). Magnification 400 \times . (B) Myocardial tissue sections obtained 28 days after STZ injection and stained by azan-mallory (A-M) method. Blue areas indicate fibrosis. Magnification 100 \times . (For abbreviations, see legend of Fig. 1.) (C) Quantification of the myocardial cell diameter using H-E stained tissue sections 28 days after STZ injection. (D) Quantification of the % of myocardial fibrosis using A-M stained tissue sections 28 days after STZ injection. (E) Western-immunoblots showing the expression of ANP, TGF β 1 and collagen III in protein lysate obtained from left ventricular tissue of transgenic TG and NTG mice 28 days after STZ injection. Equal amounts of total protein were loaded in each lane, and GAPDH protein expression was used as control. These immunoblots are representative of three separate experiments. (F–H) Quantification of ANP (F), TGF β 1 (G) and collagen III (H) protein expressions normalized with GAPDH. Densitometric analysis of immuno-reactive bands was performed using Scion Image software. The results are means \pm S.E.M. from 3–5 experiments. Open bar, NTG; closed bar, TG. * $P < 0.05$, ** $P < 0.01$ vs. N, D + L and D + T of the same genetic group. # $P < 0.05$ vs. D of NTG group.

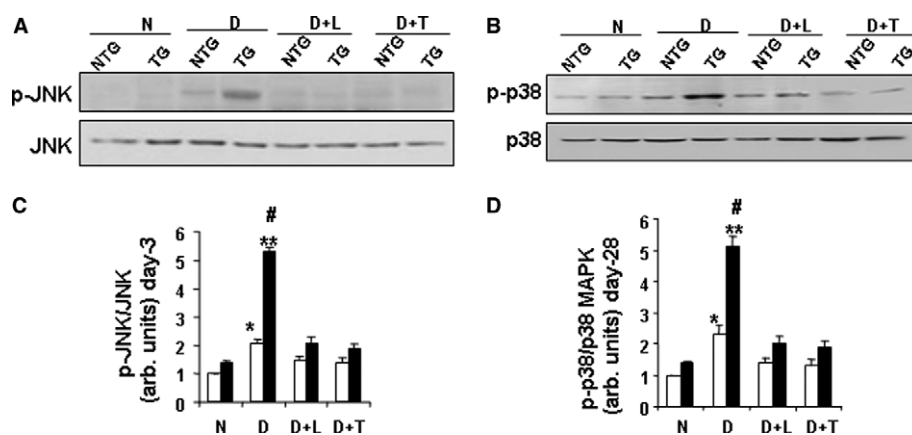


Fig. 5. Treatment with losartan and tempol attenuates MAPK signaling in the diabetic myocardium. (A) Western-immunoblots showing the myocardial expression of phospho-JNK (p-JNK) and JNK 3 days after STZ injection. (B) Western-immunoblots showing the myocardial expression phospho-p38 MAPK (p-p38 MAPK) and p38 MAPK 28 days after STZ injection. These immunoblots are representative of three separate experiments. (For abbreviations, see legend of Fig. 1.) (C) Quantification of the activation of JNK three days after STZ injection. (D) Quantification of the activation of p38 MAPK 28 days after STZ injection. Densitometric analysis of immuno-reactive bands was performed using Scion Image software, and data are represented as the mean signal intensity \pm S.E.M. from three experiments. Open bar, NTG; closed bar, TG. * $P < 0.05$, ** $P < 0.01$ vs. N, D + L and D + T of the same genetic group. # $P < 0.05$ vs. D of NTG group.

of PKC β 2 and enhanced phosphorylation of GSK3 β ser 9, (an indicator of inactive GSK3 β), which leads us to speculate that PKC β 2 may negatively regulate GSK3 β , resulting in further activation of the nuclear translocation of NFATc3 and the transcription of cardiac hypertrophic genes in the diabetic myocardium. Moreover, we showed previously that the level of phosphorylation of p38 MAPK was significantly

higher 28 days after STZ injection, and furthermore, the phosphorylation of p38 MAPK was significantly higher in DN-14-3-3 mice relative to NTG mice 28 days after STZ injection [15]. Future studies addressing the relationships among 14-3-3 protein, PKC β 2, GSK3 β and p38 MAPK may provide insights into the pathogenesis of the diabetic myocardium.

High glucose can induce the activation of p22 phox, a component of the NADPH oxidase, via de novo synthesis of DAG [28], which can further activate PKC [29]. Moreover, it has been shown that angiotensin II-induced NADPH oxidase activation is partly mediated through PKC [29,30]. Therefore, the possibility of indirect control of oxidative stress by GSK3 β through PKC also exists in the diabetic myocardium. In the present study, the number of angiotensin II-positive cardiomyocytes and the expression of AT1 receptor and p22 phox were significantly increased 3 and 28 days after STZ injection in both TG and NTG mice (Fig. 1). However, the myocardial expression of AT2 receptor did not differ among the groups (data not shown). These results show that myocardial angiotensin II and oxidative stress are elevated in the diabetic myocardium. Also, the expression of p22 phox was significantly higher in TG mice on both 3 and 28 days after STZ injection relative to that in the NTG mice at the same times. Therefore, the enhanced expression of PKC β 2 in DN-14-3-3 mice seems to be responsible for the enhanced expression of p22 phox.

It is interesting to note that both the drugs used in the present study have been reported to have the ability to alter blood glucose levels [31,32]. In this study, however, the blood glucose level remained unaltered. One possible explanation for this might be that streptozotocin induced the destruction of beta cells and depleted the levels of insulin, and moreover since tempol has been shown to improve insulin sensitivity, these drugs may not affect blood glucose per se but rather act by indirect mechanisms. However, this is still speculative and can only be verified after further investigation. We observed similar improvements after AT-1 receptor blockade as well as reduction of oxidative stress, suggesting that these events are essential for the development of diabetic cardiomyopathy and inhibiting any of these causes improvement in the molecular and pathological events in the diabetic myocardium. Tempol, a super oxide dismutase mimetic, through blockade of AT-1 receptor and reducing angiotensin II levels caused a similar reduction to Losartan in active GSK3 β /JNK-induced caspase-3 signaling 3 days after STZ injection (Fig. 6). Also, treatment with either losartan or tempol attenuated STZ-induced cardiac hypertrophy and fibrosis, through attenuating the inactivation of GSK3 β and thus preventing the nuclear translocation of NFATc3, and also attenuated the PKC β 2 and p38 MAPK signaling 28 days after STZ injection (Fig. 7). All the effects of losartan and tempol were mediated through attenuation of both myocardial angiotensin

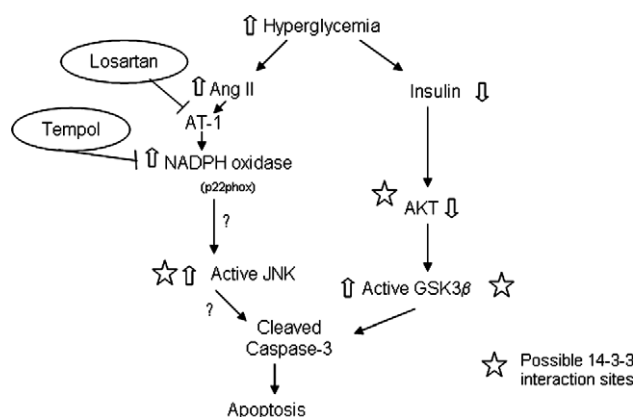


Fig. 6. An illustration of signaling pathways involved in diabetes-induced myocardial apoptosis.

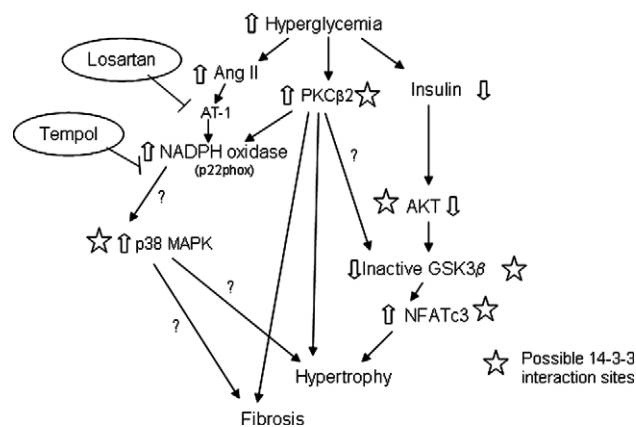


Fig. 7. An illustration of signaling pathways involved in diabetes-induced myocardial hypertrophy and fibrosis.

II and oxidative stress, as evidenced by the reduction in the number of angiotensin II-positive cardiomyocytes and the reduction of the expression of p22 phox (Fig. 1). A primary mediator of angiotensin II stimulated collagen production in vitro is thought to be TGF β 1 [33]. In this study, treatment with either losartan or tempol attenuated the expression of TGF β 1 and collagen III (Fig. 4). Collectively, our results suggest that angiotensin II via AT1 receptor and oxidative stress play a major role in diabetes-induced myocardial apoptosis, hypertrophy and fibrosis, and that the treatment with either an AT1 receptor blocker or with an antioxidant will be beneficial for diabetic cardiomyopathy. However, further studies are required to verify this. Moreover, based on our results we could not differentiate between the effect of treatment with losartan and tempol. These results suggest that the relationship among angiotensin II, oxidative stress and GSK3 β should be studied. Further studies are warranted to clarify the dual role of GSK3 β in the diabetic myocardium.

In conclusion, our results strongly suggest the role of GSK3 β signaling in the diabetic myocardium, where GSK3 β is more activated at the early stage of experimental diabetes, which at least in part contributes to the myocardial apoptosis. Moreover, the inactivation of GSK3 β and nuclear translocation of NFATc3 probably at least in part contributes to the myocardial hypertrophy and fibrosis at later stages of experimental diabetes. Inactivation of 14-3-3 protein in DN-14-3-3 mice exacerbates cardiac apoptosis and hypertrophy, effects which are at least in part mediated through enhanced activation of GSK3 β and GSK3 β phosphorylation-mediated enhanced nuclear translocation of NFATc3. Also, treatment with losartan or tempol attenuates experimental diabetes-induced myocardial apoptosis, hypertrophy and fibrosis through attenuating the signaling of GSK3 β , p38 MAPK, JNK and PKC β 2.

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